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New Karyological and Morphometric Data on Poorly Known *Bufo surdus* and *Bufo luristanicus* in Comparison with Data of Diploid Green Toads of the *Bufo viridis* Complex from South of Iran

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Abstract Previous studies on the *Bufo viridis* complex, which is distributed broadly across Iran, are incomplete and restricted to a few regions or a few samples. In this paper a new detailed study on the *B. viridis* complex in southern of Iran (from West to East) is presented. The analysis of 18 morphometric characters with univariate and multivariate methods reveals significant differences between three members of the *B. viridis* complex namely *B. variabilis*, *B. luristanicus*, and *B. surdus* distributed in southern part of Iran. Our result help to resolve an old taxonomic problem about *B. surdus* subgroup (taxa closely related to *B. surdus*) confirming that *B. luristanicus* and *B. surdus* are distinct species. Moreover, for the first time we report and describe karyotype details of *B. luristanicus* and *B. surdus* which confirmed that they are diploid. Karyological studies demonstrate that all toads from three mentioned species have 2n = 22 chromosomes. These chromosomes are arranged into two groups. First group has six large chromosomes and the second group is composed of five small chromosomes. These chromosomes are metacentric or submetacentric. The number of submetacentric chromosomes is different in three mentioned species of *B. viridis* complex. Neither sexual heteromorphism, nor secondary constriction was observed in any pairs of chromosomes.

Keywords *Bufo viridis* complex, taxonomy, *Bufo surdus*, *Bufo luristanicus*, *Bufo variabilis*, karyotype, morphometry, Iran

1. Introduction

Green toads (*Bufo viridis* complex) are one of the prominent anuran species complexes which are broadly spread in the Palearctic region. Since *B. viridis* was described by Laurenti in the 18th century, considerable morphological variation has been observed for green toads, resulting in the description of numerous forms as species and subspecies (Stöck *et al.*, 2001a, 2005). The complexity of this species complex and variation in cytogenetic forms in Asia is more than Europe and Africa (Batista, 2006). So far, all three ploidy levels (diploid, triploid and tetraploid) have been reported from Asia (Stöck *et al.*, 2001b). Recent cytogenetic studies show that in addition to the diploid forms that have been reported from Iran, *B. oblongus* species distributed in east of Iran is tetraploid (Stöck *et al.*, 2001b, 2003, 2005, 2006).

Despite significant progress in this area, the taxonomy of the *B. viridis* complex is still unknown. Ploidy level of many populations is not recognized and the relationships among members of this group are uncertain and need further investigations (Borkin *et al.*, 2000). It has been indicated before that there are three taxa closely related

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to *B. surdus*, namely *B. luristanicus, B. surdus surdus* and *B. surdus annulatus,* which together are the *B. surdus* subgroup (e.g. Baloutch and Kami, 1995; Stöck *et al.*, 2001a). These taxa occur in Iran and have many characters in common, including a relatively small body size, extremely small or absent visible tympana and nearly squared parotoid glands. Inger (1972) placed these taxa in the *B. viridis* complex while Stöck *et al.* (2001a) believe the *B. surdus* subgroup does not belong to the *B. viridis* complex. Taxonomic status of *B. surdus* subgroup is still unknown. Moreover, ploidy levels and karyotype details of members of *B. surdus* subgroup are unclear and need further study.

Bufo luristanicus, *B. surdus* (with two subspecies) and *B. variabilis* are three members of *B. viridis* complex distributed in south, south west and south east of Iran. *B. luristanicus* is endemic to Iran and inhabits the Zagrous Mountains. *B. surdus surdus* exists in south and south east of Iran and southwest of Pakistan. *B. surdus annulatus* is exclusively found in its type locality in southwestern of Iran, 80 km south of Shiraz, Fars Province (Schmidtler and Schmidtler, 1969). *B. variabilis* is found in the western and central parts of Iran.

Recent studies of the green toad complex in the Palearctic include only a few samples from limited parts of the vast territory of Iran (Stöck *et al.*, 2001a, 2001b, 2005, 2006; Borkin *et al.*, 2000; Litvinchuk *et al.*,2007; Javari and Torki, 2009; Degani *et al.*, 2013). As a result, the available data on *B. viridis* complex in Iran is incomplete, and the taxonomic status of such species is unknown or uncertain.

The aim of this paper is to present a new detailed study of the *B. viridis* complex in the southern region of Iran and provide helpful information on distribution, morphology, morphometry and karyotype of *B. viridis* complex, so that more precise taxonomic decisions can be adopted.

2. Materials and Methods

2.1 Sampling A total of 47 specimens of *B. viridis* complex were collected from various regions in the southern part of Iran, from March 2012 to April 2013. Sampling information is given in Table 1 and Figure 1.

2.2 Morphometry Eighteen morphometric characters were measured (Baloutch and Kami, 1995; Stöck *et al.*, 2001a) using a digital caliper with an accuracy of 0.01mm. SPSS 16 was used for statistical analysis. Note that only adult toads were included in this study. The measured characters are as follows:

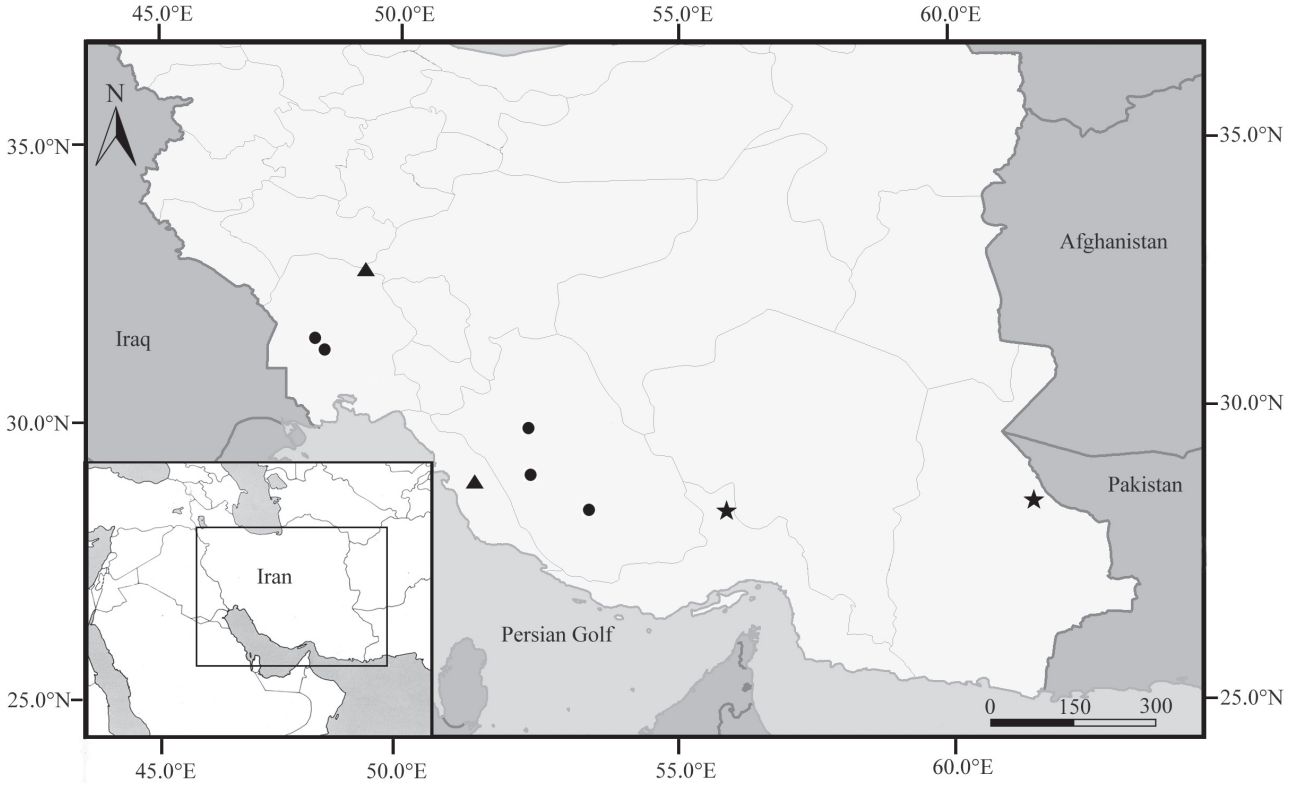

Figure 1 Map of Iran showing sampling localities of *Bufo viridis* complex. ●: *B. variabilis*; ▲: *B. luristanicus*; ★ : *B. surdus*.

Table 1 Localities of toads studied in this work.

Taxon	Locality	N	Coordinates	Elevation
Bufo. variabilis	Kourosh neighborhood, Ahvaz, Khuzestan Province	7	31°23' N 48°44.7' E	12 m
B. variabilis	Lavi Spring near Khammat village, Choqa-zanbil, Khuzestan Province	8	32°31' N 48°32' E	70 m
B. variabilis	Firoozabad road, Mehkuh Olia village (80 km south of Shiraz), Fars Province	6	29°1' N 52°26' E	1685 m
B. variabilis	Phase 4 of Sadra town, Shiraz, Fars Province	6	29°49' N 52°26' E	1820 m
B. variabilis	Jahangirkhani village, 10 km north of Hosseiniye region, Andimeshk, Khuzestan Province	1	32°45' N 49°32' E	475 m
B.luristanicus	Jahangirkhani village, 10 km north of Hosseiniye region, Andimeshk, Khuzestan Province	11	32°45' N 49°32' E	475 m
B. luristanicus	Bushehr, Bushehr Province	2	28°58' N 51°17' E	–
B.surdus surdus	Sangan region, Khash, Sistan and Baluchestan Province	4	28°36' N 61°18' E	1690 m
B.surdus surdus	Haji Abad, Hormozgan Province	1	28°19' N 55°54' E	933 m
B. variabilis	Barous Plain 11km east of Mobarakabad, Qir-Jahrom road, Fars Province	1	28°21' N 53°26' E	765 m

1- Snout-vent length (SVL), 2- head length (HL), 3- distance between tip of snout and anterior corner of eye (ESD), 4- internarial distance (IND), 5- upper eyelids distance (UED), 6- width of upper eyelid (UEW), 7- horizontal diameter of eye (ED), 8- tympanic diameter (DT), 9- thigh length (THL), 10- length of tibia (TL), 11- tarsus length: from the heel to the proximal edge of the inner metatarsal tubercle (TAL), 12- foot length: from proximal edge of the inner metatarsal tubercle to tip of 4th toe (FL), 13- length of first toe (LFT), 14- length of inner metatarsal tubercle (LMT), 15- length of parotoid gland (PL), 16- width of parotoid gland (PW), 17- distance between parotoid glands (PD), and 18- distance between nostril and anterior corner of eye (NED).

2.3 Chromosome preparation Specimens were transferred to lab alive. Toads were injected intraperitoneally with 0.3% colchicine solution for 12–16 hours before being sacrificed. Bone marrow was extracted and placed in 0.075 M KCL hypotonic solution. Then the solution was kept in room temperature for 45–55 minutes and the procedure described in Schmid (1978) was followed. Slides were stained with 4% Giemsa for 10–15 minutes, were studied with a microscope and well spread metaphase was photographed. Based on Levan *et al*. (1964) classification, chromosome types were determined and arranged to obtain a chromosomal set. Karyotype Analysis 1.2 software was used for morphometric measurements of each chromosome.

3. Results

3.1 Morphometric analysis

Univariate statistics The mean values and standard deviations of all morphometric characters were significantly different in the three taxa of *B. viridis* complex.

Our results (Table2) show that specimens of *B. variabilis* have the largest body length (SVL= 70.48 ± 8.73 mm) and *B. luristanicus* has the smallest body length (50.70 ± 3.36 mm) among the three taxa. *B. variabilis* has the largest values for all other morphometric characters except UED. *B. surdus* has the largest values For UED. Similarly, *B. luristanicus* owns the smallest amount in all morphometric characters except DT and LFT. DT was not observed for *B. surdus*. LFT of *B. luristanicus* was larger than that of *B. surdus* (3.52 ± 0.40 mm compared to 3.23 ± 0.41 mm). In this analysis, *B. surdus* stands in between the other two groups (Table 2) for all morphometric characters (except DT and LFT). Also analysis of variance (ANOVA) showed significant differences between the three groups in all measured characters (P<0.05). Character variation range in *B. variabilis* was considerably higher than the other groups (For example Character variation range or Min-Max of SVL in *B. variabilis* (41.08 mm–85.21 mm) was considerably higher than *B. surdus* (56.4 mm–66.04 mm) and *B. luristanicus* (46.22 mm–58.65 mm)).

It is notable that tympanic membrane was not visible in all collected samples belonging to *B. surdus* in this work , while all *B. luristanicus* samples had externally visible tympanic membrane. This membrane in *B. luristanicus* was smaller than *B. variabilis* (Table 2).

Multivariate statistics Discriminant analysis was used to describe functions that maximize the probability of correct classification of specimens to their original population. A stepwise discriminate analysis with eighteen morphometric traits reclassified 100% of all samples correctly into the three groups; *B. variabilis*, *B. luristanicus* and *B. surdus*. Each species was placed 100% in its expected group (see Table 3).

Based on Wilks' Lambda, both functions are significant (P < 0.05). First function represented 75.7% of variance and the second one showed 24.3% of variance.

Table 2 Morphometric characters of *Bufo viridis* complex (measurments in mm).

Characters	*Bufo variabilis* (n=29)		*B. surdus* (n=5)		*B. luristanicus* (n=13)		*P*
	Mean ± SD	Min / Max	Mean ± SD	Min / Max	Mean ± SD	Min / Max	
SVL	70.48 ± 8.73	41.08–85.21	60.73 ± 4.35	56.4–66.04	50.70 ± 3.36	46.22–58.65	0
HL	20.29 ± 1.58	16.95–24.21	17.09 ± 1.19	15.56–18.32	13.54 ± 0.86	11.92–14.79	0
ESD	9.25 ± 0.82	6.99–10.54	7.38 ± 0.81	6.6–8.53	5.86 ± 0.35	5.03–6.47	0
IND	4.31 ± 0.54	3.02–5.37	3.68 ± 0.28	3.27–3.96	2.85 ± 0.25	2.49–3.26	0
UED	5.44 ± 0.68	4.21–6.74	5.55 ± 1.06	4.2–6.92	3.91 ± 0.31	3.29–4.53	0
UEW	5.94 ± 0.61	4.5–7.04	5.44 ± 0.89	3.99–6.38	4.61 ± 0.31	4.26–5.29	0
ED	7.10 ± 0.67	5.9–8.18	6.15 ± 0.56	5.54–6.63	5.31 ± 0.36	4.89–6.05	0
DT	3.80 ± 0.46	2.76–4.54	0 ± 0	0–0	2.15 ± 0.34	1.74–2.69	0
THL	26.69 ± 3.08	19.38–33.42	23.65 ± 1.83	21.83–26.42	19.48 ± 1.06	17.91–20.98	0
TL	26.90 ± 2.76	22.52–32.11	24.38 ± 2.13	21.77–26.29	20.01 ± 1.53	17.28–22.14	0
TAL	17.32 ± 1.90	13.38–20.99	14.62 ± 1.52	12.31–16.15	12.06 ± 0.83	10.56–13.46	0
FL	30.41 ± 2.89	24.67–35.42	26.67 ± 1.89	23.88–28.4	21.65 ± 1.16	19.15–23.07	0
LFT	5.29 ± 0.69	4.06–7	3.23 ± 0.41	2.54–3.58	3.52 ± 0.40	3.02–4.51	0
LMT	3.64 ± 0.58	2.41–4.68	3.06 ± 0.28	2.83–3.53	2.17 ± 0.40	1.62–3	0
PL	15.06 ± 1.87	11.1–18.61	7.61 ± 1.27	5.77–8.88	5.15 ± 0.64	3.99–6.32	0
PW	7.37 ± 1.29	4.97–9.93	7.06 ± 0.97	5.45–7.86	4.51 ± 0.68	3.24–5.81	0
PD	9.50 ± 1.14	5.21–11.02	9.45 ± 2.93	4.88–13	7.99 ± 1.36	4–9.5	0.011
NED	5.48 ± 0.39	4.76–5.97	5.07 ± 0.82	4.43–6.23	3.81 ± 0.32	3.18–4.5	0

Table 3 Classification results of discriminate analysis with eighteen morphometric characters.

		Group	Predicted Group Membership			Total
			Bufo variabilis	*B. surdus*	*B. luristanicus*	
Original	Count	*Bufo variabilis*	29	0	0	29
		B. surdus	0	5	0	5
		B. luristanicus	0	0	13	13
	%	*B. variabilis*	100	0	0	100
		B. surdus	0	100	0	100
		B. luristanicus	0	0	100	100

Five of eighteen morphometric characters had the highest values of standardized canonical discriminant coefficients (Table 4).

Also Scatter plot of canonical functions show a clear separation between these three groups (Figure 2).

3.2 Karyotype analysis In this study, we reported and described karyotype of *B. luristanicus* for the first time. This species was diploid and had 22 chromosomes (Figures 3 and 4).

Chromosomes were divided into two major groups. First group consists of 6 large chromosomes and the second group contains 5 small chromosomes. Morphometric data such as length of short arm, length of long arm and other parameters are demonstrated in Table 5. *B. luristanicus* karyotype consists of 8 pairs of metacentic and 3 pairs of submeatacentric chromosomes. The 4th, 5th and 7th pairs of chromosomes were submetacentric, therefore metacentic and submeatacentric chromosomes were observed in both major groups. Related idiogram is shown in Figure 5.

Result of karyotype of *B. surdus* showed a set of 2n= 22 chromosomes (Figures 6 and 7). These chromosomes were arranged into two groups. First group consisted of 6 large chromosomes and the second one had 5 small chromosomes. Morphometric data of *B. surdus* is shown in Table 6.

The 1st, 4th, 5th and 7th pairs of chromosomes were submetacentric and the remaining pairs were metacentric. Therefore, metacentic and submetacentric chromosomes were observed in both major groups. The idiogram of this species is demonstrated in Figure 8.

Karyotype of *B. variabilis* from four localities of south Iran showed that this subspecies was diploid and had 2n = 22 chromosomes (Figures 9 and 10). Two major groups could be distinguished. The first group consists of six large chromosomes and the second group has five small chromosomes.

Karyotype of samples belonging to Mehkuh, Shiraz and Ahvaz exhibited that 4th and 7th pairs of chromosomes were submetacentric but in samples from Choqa-zanbil 4th and 9th pairs of chromosomes were submetacentric.

Table 4 Standardized canonical discriminant function coefficients for *Bufo viridis* group. Five of eighteen characters were selected.

Characters	Function	
	1	2
ESD	0.135	0.652
DT	0.55	–1.034
TL	–1.076	–0.387
FL	0.494	0.542
PL	0.906	0.45

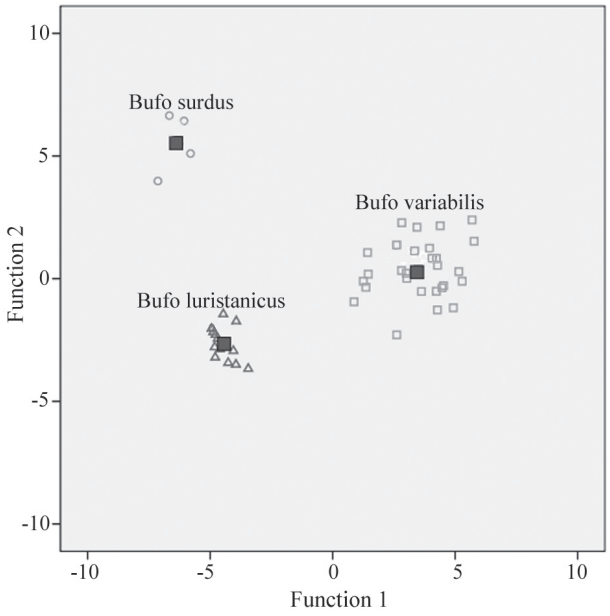

Figure 2 Scatter plot of canonical discriminant functions of eighteen morphometric traits of *Bufo viridis* complex

Morphometric analysis of chromosomes of *B. variabilis* samples is shown in Table 7 and Table 8.

Submetacentric chromosomes exist in both major groups. Related idiograms of *B. variabilis* belonging to four mentioned regions are presented in Figures 11 and 12.

Other data from field Unfortunately, the attempts to get *B. surdus annulatus* subspecies from its type locality (80 km south of Shiraz, 5 km north of Mehkuh, Fars Province) in spring and summer seasons failed. This region is the only place for this subspecies that has been identified so far. Amphibians that were found in this area were *Pelophylax ridibundus*, *Hyla savignyi* and *B. variabilis*. Therefore, only *B. surdus surdus* subspecies were included in this research. Unfortunately, our efforts to obtain more specimens of *B. surdus surdus* to increase the sample size were not successful. One reason was that Sistan and Baluchestan (which is a large part of distribution areas of this subspecies) is an unsafe place to work specially in the border areas with the two neighboring countries and the other reason is that distribution range of this subspecies might have changed over time. For example, previously *B. surdus surdus* had been reported from Rudan (Deh Barez) in Hormozgan Province (Stöck *et al.*, 2006), but we could not get this subspecies from Rudan. The area was occupied with an oriental species named *Duttaphrynus olivaceus*. Similarly, Zareian *et al.* (2012) found only one sample of *B. surdus surdus* from Gorm Mountain in Fars, south west of Iran.

Figure 3 *Bufo luristanicus* from Hosseiniye region of Andimeshk, Khuzestan Province. Photo by F. Fakharzadeh.

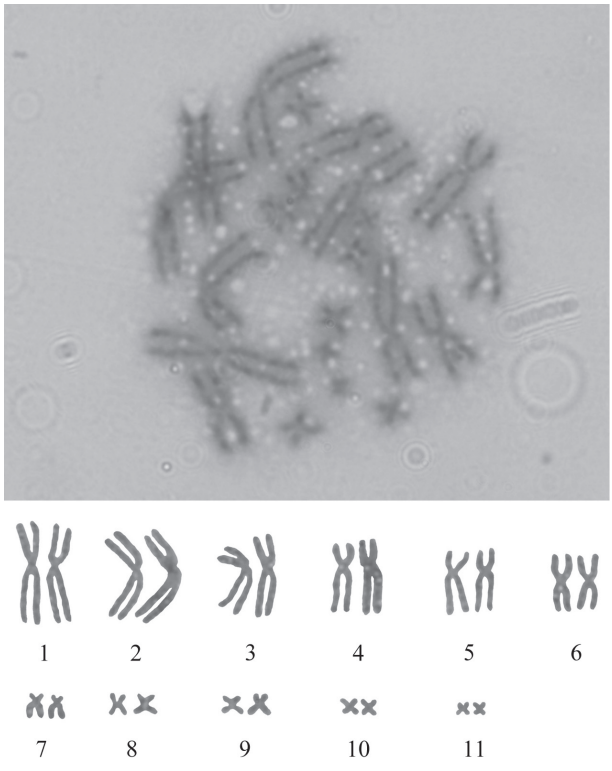

Figure 4 Karyotype of *Bufo luristanicus* from Hosseiniye region of Andimeshk, Khuzestan Province.

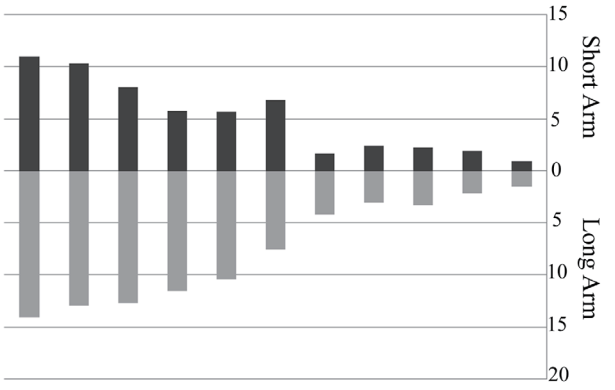

Figure 5 Idiogram of *Bufo luristanicus* based on centromere position.

Figure 6 *Bufo surdus* from Sangan region of Khash. Photo by F. Fakharzadeh.

Bufo luristanicus samples collected from a river near Hosseiniye region of Andimeshk, Khuzestan Province (a place near the type locality of this species) were sympatric with *B. variabilis* at the same region.

4. Discussion

4.1 Morphometry and morphology The univariate and multivariate analyses of the morphometric characters used in this study revealed that the morphometric traits can clearly and completely discriminate *B. luristanicus, B. variabilis* and *B. surdus* taxa from each other and confirmed that they are separate species. Moreover, as mentioned in this study (Section 3.1) tympanic membrane is a good morphological character to distinguish these taxa from each other. *B. surdus* subgroup is distributed in southern region of Iran, which includes *B. surdus surdus*, *B. surdus annulatus,* and *B. luristanicus*. They have many common characters such as small body size, lack of visible eardrum or it may be very tiny and also all have square parotoid glands.

Schmidtler and Schmidtler (1969) introduced *annulatus* and *luristanicu*s as subspecies of *B.surdus,* but Mertens (1971) introduced *B. luristanicus* as a separate species. Stöck *et al.* (2001a) considered these two species as a separate species, because of lack of visible eardrum in *B. surdus* and its existence in *B. luristanicus*. Also Anderson (1985) and Baloutch and Kami (1995) considered *B.*

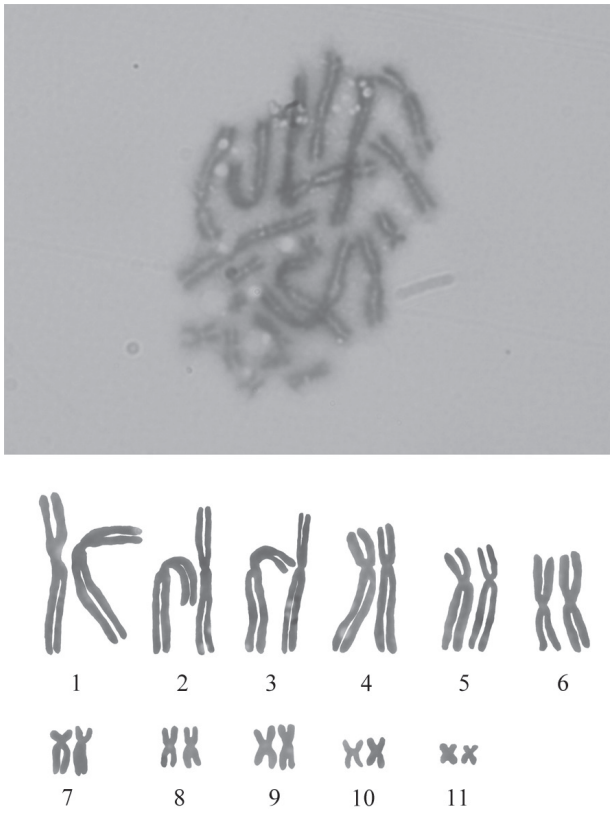

Figure 7 karyotype of *Bufo surdus* from Khash, Sistan and Baluchestan Province.

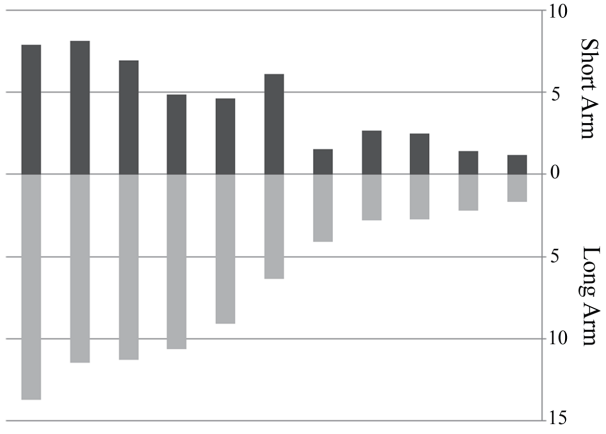

Figure 8 Idiogram of *Bufo surdus* based on centromere position.

Table 5 Morphometric data of *Bufo luristanicus* chromosomes.

Chromosome No.	Short arm (p)	Long arm (q)	Total length (q+p)	Arm ratio (q/p)	Morphology
1	11.03	14.07	25.1	1.3	M
2	10.36	12.87	23.23	1.2	M
3	8.1	12.67	20.77	1.6	M
4	5.75	11.49	17.23	2	SM
5	5.74	10.36	16.1	1.8	SM
6	6.87	7.56	14.43	1.1	M
7	1.67	4.19	5.86	2.5	SM
8	2.45	3.07	5.53	1.3	M
9	2.26	3.26	5.52	1.4	M
10	1.96	2.12	4.09	1.1	M
11	0.98	1.51	2.49	1.5	M

Table 6 Morphometric data of *Bufo surdus* chromosomes.

Chromosome No.	Short arm (p)	Long arm (q)	Total length (q+p)	Arm ratio (q/p)	Morphology
1	7.9	13.74	21.75	1.7	SM
2	8.18	11.47	19.65	1.4	M
3	6.97	11.3	18.27	1.6	M
4	4.89	10.63	15.52	2.2	SM
5	4.62	9.1	13.72	2	SM
6	6.15	6.37	12.51	1	M
7	1.53	4.11	5.64	2.7	SM
8	2.71	2.78	5.49	1	M
9	2.48	2.72	5.21	1.1	M
10	1.45	2.2	3.64	1.5	M
11	1.18	1.66	2.84	1.4	M

luristanicus as distinct species. Afrasiab and Ali (1988) reported *B. surdus* from Iraq, but it was probably *B. luristanicus*. Torki and Javari (2009) believed that *B. luristanicus* is a subspecies of *B. surdus* and rejected the existence of *luristanicus* as a separate species. The results of present research are in total agreement with Mertens (1971), Stöck *et al.* (2001a), Anderson (1985), Baloutch and Kami (1995) statements. Despite Torki and Javari (2009) that indicated the well-develop spiny tubercles on dorsal surface of *B. luristanicus* and lack of this trait in *B. variabilis*, our observation shows the existence of this trait in the majority of *B. luristanicus*, *B. variabilis* and *B. surdus* samples.

The results presented in this work can resolve an old taxonomic problem in *B. surdus* subgroup. This study confirms that *B. surdus* and *B. luristanicus* are separate species. Moreover, the existence of externally visible tympanic membrane in all *B. luristanicus* samples and its absence in all *B. surdus* samples verify it to be a proper morphological character to distinguish these two species.

Therefore, common morphological traits in *luristanicus* and *surdus* (such as parotoid glands shape) are illusive that can be misleading even for a specialist at first glance. Our data confirm the existence of two distinct green toads in Zagrous Mountains of Iran and this conclusion agrees with the statements of Stöck *et al.* (2001b) and Eiselt and Schmidtler (1973).

On the other hand, most of *B. surdus surdus* samples collected in this study belong to Khash, Sistan and Baluchestan, the nearest available location in Iran to the type locality of this subspecies, which is Baluchestan, Pakistan (Boulenger,1891). The holotype is an adult male specimen without visible tympanic membrane and SVL is 67 mm (Stöck *et al.*, 2001a). Range of SVL for our samples (See Table 2) is 56.4 to 66.04 mm. Also they are without visible tympanic membrane. So, in comparison to the holotype, our collected samples of this subspecies are slightly smaller. *B. surdus surdus* is distributed in Kerman, Sistan and Baluchestan and Hormozgan (Haji Abad) provinces (Baloutch and Kami 1995; Stöck *et al.*, 2001a). The type locality of *B. surdus annulatus* is 80 km south of Shiraz, 5 km north of Mehkuh), Fars province (Schmidtler and Schmidtler, 1969). So far, only one specimen of this subspecies has been found from type locality in 1968. As described earlier, our attempt to find *B. surdus annulatus* from its type locality failed.

According to Schmidt (1955) the type locality of *B. luristanicus* is Shahbazan at kilometer 324 of the trans-

Table 7 Morphometric data of *Bufo variabilis* chromosomes from two localities of Fars. A: Mehkuh; B: Shiraz.

Chromosome No.	A					B				
	Short arm (q)	long arm (p)	Total length (q+p)	Arm ratio (q/p)	Morphology	Short arm (q)	long arm (p)	Total length (q+p)	Arm ratio (q/p)	Morphology
1	12.95	15.28	28.2	1.2	M	10.54	11.19	21.7	1.1	M
2	12.46	15.14	27.6	1.2	M	8.84	11.7	20.5	1.3	M
3	10.74	14.46	25.2	1.3	M	6.88	10.44	17.3	1.5	M
4	7.67	14.62	22.3	1.9	SM	5.65	10.61	16.3	1.9	SM
5	9.25	10.4	19.7	1.1	M	5.99	6.71	12.7	1.1	M
6	7.37	10.01	17.4	1.4	M	5.25	7.31	12.6	1.4	M
7	3.37	6.31	9.7	1.9	SM	2.26	4.13	6.4	1.8	SM
8	3.76	4.72	8.5	1.3	M	2.41	3.23	5.6	1.3	M
9	3.43	4.49	7.9	1.3	M	2.15	3.07	5.2	1.4	M
10	2.91	3.1	6	1.1	M	1.54	2.44	4	1.6	M
11	2.28	3.31	5.6	1.4	M	1.5	2.16	3.7	1.4	M

Table 8 Morphometric data of *Bufo variabilis* chromosomes from two localities of Khuzestan. A: Ahvaz; B: Choqa-zanbil.

Chromosome No.	A					B				
	Short arm (q)	Long arm (p)	Total length (q+p)	Arm ratio (q/p)	Morphology	Short arm (q)	long arm (p)	Total length (q+p)	Arm ratio (q/p)	Morphology
1	12.5	13.63	26.1	1.1	M	11.7	19.14	30.8	1.6	M
2	9.63	12.79	22.4	1.3	M	13.26	15.74	29	1.2	M
3	8.25	12.14	20.4	1.5	M	11.39	17.13	28.5	1.5	M
4	6.75	12.25	19	1.8	SM	8.1	14.46	22.6	1.8	SM
5	6.85	7.37	14.2	1.1	M	8.6	12.45	21	1.4	M
6	5.11	7.87	13	1.5	M	8.08	10.62	18.7	1.3	M
7	2.32	4.72	7	2	SM	4.08	5.74	9.8	1.4	M
8	2.54	3.97	6.5	1.6	M	4.03	5.62	9.7	1.4	M
9	2.61	3.38	6	1.3	M	2.8	6.82	9.6	2.4	SM
10	2.29	2.58	4.9	1.1	M	3.28	3.62	6.9	1.1	M
11	1.5	2.2	3.7	1.5	M	2.44	3.02	5.5	1.2	M

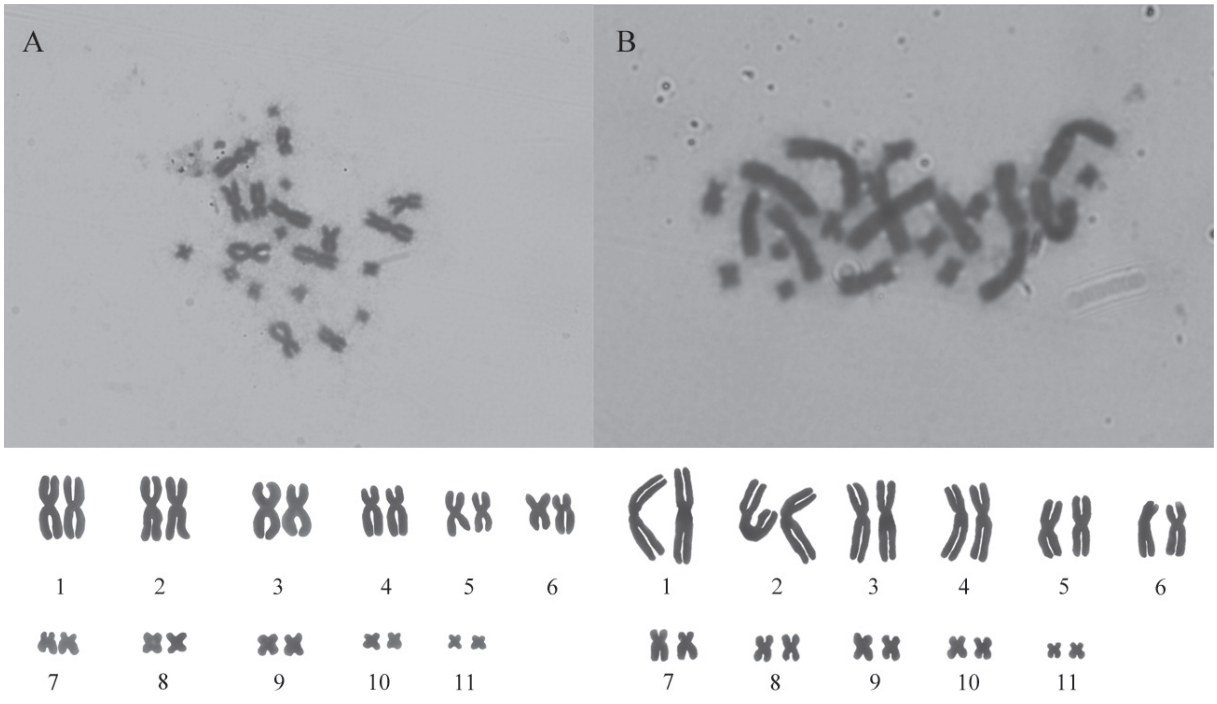

Figure 9 Karyotype of *Bufo variabilis* from two localities of Fars province. A: Mehkuh; B: Shiraz.

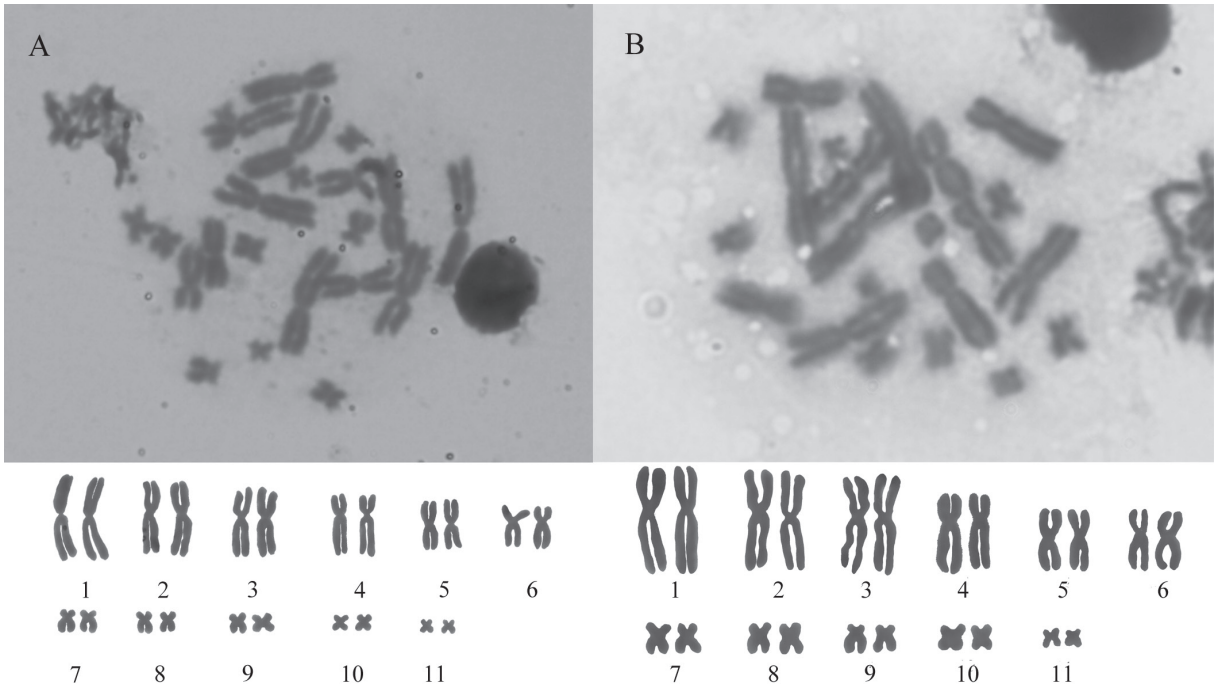

Figure 10 Karyotype of *Bufo variabilis* from two localities of Khuzestan province. A: Ahvaz; B: Choqa-zanbil.

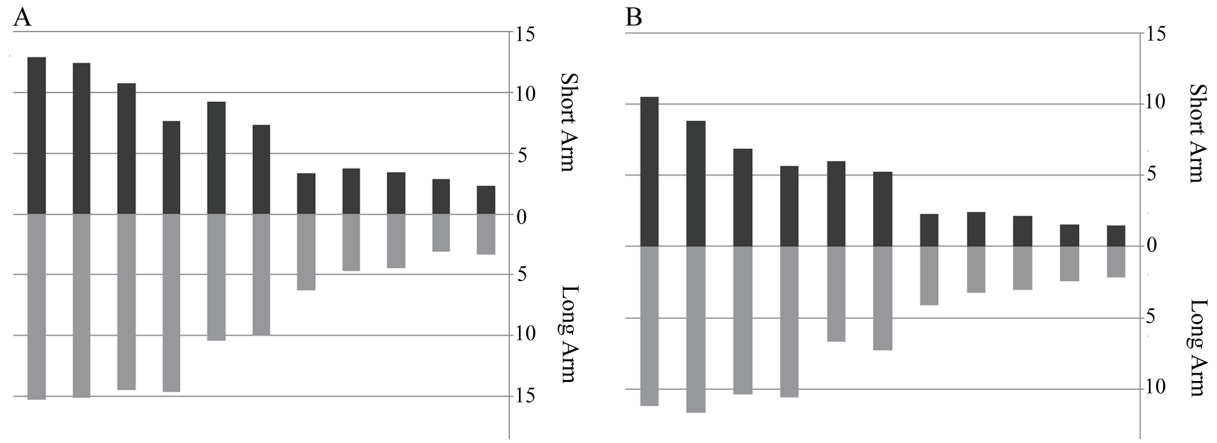

Figure 11 Idiogram of *Bufo variabilis* based on centromere position from two localities of Fars. A: Mehkuh; B: Shiraz.

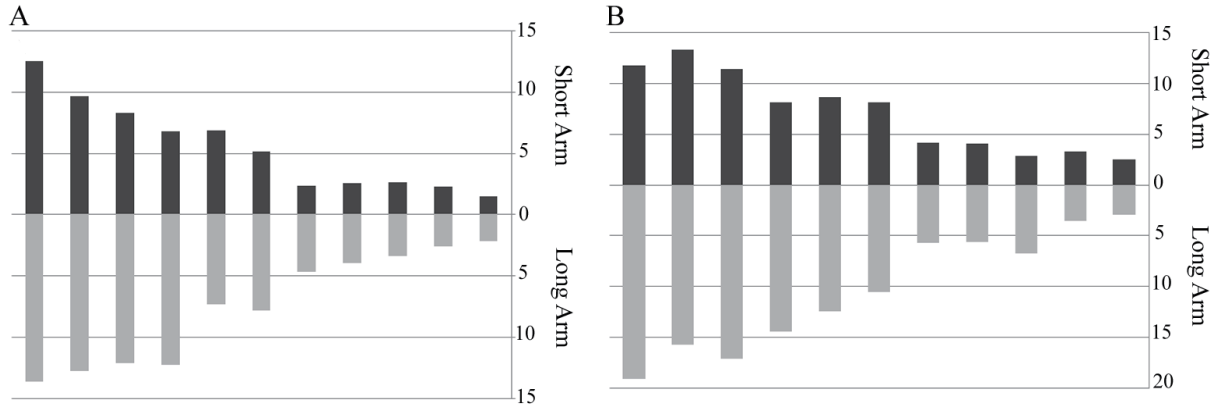

Figure 12 Idiogramof *Bufo variabilis* based on centromere position from two localities of Khuzestan. A: Ahvaz; B: Choqa-zanbil.

Iranian railway, Lorestan province. 52 km as the crow flies NE of Dizful. But our researches show that there are two localities in Iran which called Shahbazan. One of them is located in lorestan Province without any railway near it but the other one (Shahbazan station) is located near Andimeshk and in 52 km as the crow flies NE of Dizful in Khuzestan Province and there is a railway here. Therefore we concluded that probably the type locality of *B. luristanicus* is near Andimeshk and Dizful in Khuzestan Province not in Lorestan province. Most of the collected samples of this species are from Hosseiniye region of Andimeshk. So this region is very close to the type locality of *B. luristanicus*. The Holotype is a male that its SVL is 51.8 mm. In comparison, range of our samples for SVL is 46.22 to 58.65 mm (See Table 2). So far *B.luristasnicus* has been reported from Fars, Lorestan and Khuzestan provinces. *B. variabilis* is distributed in western half and central part of Iran. Type locality of *B. variablis* is far from Iran and located in Germany (Pallas, 1777).

4.2 Karyotype For the first time karyotypic data for *B. luristanicus* and *B. surdus* were reported. All collected green toads that belong to three mentioned species are diploid and have 22 chromosomes. Chromosomes are arranged into two groups. First group has six large chromosomes and the second group composes of five small chromosomes. These chromosomes are metacentric or submetacentric. Such pattern is highly conserved in most species of genus *B.* (e.g. Borkin *et al.*, 2007; Bogart, 1972). In the examined samples of southern region of Iran, polyploid toads were not found. So far polyploid toads have been reported only by Stöck *et al.* (2001b, 2005, 2006) from east of Iran. Stöck *et al.* (2001b) stated that the deserts of Central Iran seem to separate the polyploids in the East of Central Iran from the diploids in the West. Therefore if their conclusion is correct, polyploid toads must be restricted to Eastern part of Iran.

According to Stöck *et al.* (2001a) the results obtained from DNA flow cytometry show that both *B. luristanicus* and *B. surdus* are diploid. Our karyological studies that have been done on *B. luristanicus* and *B. surdus* also confirm this conclusion.

Our study shows no sexual heteromorphism in any pairs of chromosomes. The uniformity of sex chromosomes among toads is accepted and indicated in many papers (Miura, 1995; Kasahara *et al.*, 1996; Cavallo *et al.*, 2002; Borkin *et al.*, 2007; Siripivasing *et al.*, 2008; Fakharzadeh *et al.*, 2009; Al-Shehri and Al-Saleh, 2010).

The number of submetacentric chromosomes is different in three mentioned species of *B. viridis* complex. Karyotype of *B. surdus* contains 4 pairs of submetacentric chromosome (1st, 4th, 5th and 7th pairs of chromosomes) while in *B. luristanicus* 3 pairs of chromosomes (4th, 5th and 7th pairs of chromosomes) are submetacentric and *B. variabilis* from four mentioned localities has only 2 pairs of submetacetric chromosomes (4th and mostly 7th pairs of chromosomes).

Our findings on karyotype of *B. variabilis* species from four mentioned localities exhibited no secondary constriction. Same results are indicated in other studies that used Giemsa-stained method (e.g. Borkin *et al.*, 2007; Masik *et al.*, 1976; Borkin *et al.*, 1986a; Roth and Ráb, 1986). In Giemsa-stained karyotype of diploid *B. viridis* from Kerman (Stöck *et al.*, 2001a, 2005), the chromosome pair 6 had a secondary constriction on its long arms, which is in contrast to the present study.

5. Conclusion

The results of this paper verify the existence of three independent species of *B. viridis* complex, namely *B. luristanicus, B. surdus* and *B. variabilis,* in southern region (south, southeast and southwest) of Iran. In the present study for the first time karyotype details of *B. surdus* and *B. luristanicus* were reported. It was confirmed that both species are diploid and have 22 chromosomes. Moreover, it was explored that the number of submetacentric chromosomes is different in the three taxa of *B. viridis* complex.

These results can resolve an old taxonomic problem about *B. surdus* subgroup (taxa closely related to *B. surdus*). In summary *B. luristanicus* and *B. surdus* are distinct species and common morphological traits in *luristanicus* and *surdus* are seductive.

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References

Afrasiab S. R., Ali H. A. 1988. A new record of toad *Bufo surdus,* Boulenger (Amphibia, Bufonidae) from Iraq with preliminary key for Iraqi Amphibia. Bull Iraq Nat Mus, 8(1): 115–123

Al-Shehri A. H., Al-Saleh A. A. 2010. First report of the karyotype of *Bufo dhufarensis* from Saudi Arabia. J King Saud Univ, Science , 22: 73–76

Anderson, S. C. 1985. Amphibians. In Yarshater E. (Ed.), Encyclopedia Iranica. Vol. 1, Fascicle 9: 987–990

Baloutch M., Kami H. G. 1995. Amphibians of Iran. Tehran, Iran:

Tehran University Press, 177 pp (In Persian)

Batista V. , Carranza S. ,Carretero M. A., Harris D. J. 2006 Genetic variation within *Bufo viridis:* Evidence from mitochondrial 12s and 16s rRNA DNA sequences. Bull Soc Catalana Herpetol Barcelona, 17: 24–33

Bogart J. P. 1972. Karyotypes. In Blair W. F. (Ed.), Evolution in the Genus Bufo. Austin: University of Texas Press, 171–195

Borkin L. J., Caune I. A., Pisanetz E. M., Rozanov Y. M. 1986. Karyotype and genome size in the *Bufo viridis* group. In Roček Z. (Ed.), Studies in Herpetology, Charles University, Prague, 137–141

Borkin L. J., Rozanov J. M., Litvinchuk S. N. 2000. Nuclear DNA content in some green toads (*Bufo viridis* complex) of Turkey and Iran. Russ J Herpetol, 7(3): 171–180

Borkin L. J., Shabanov D. A., Brandler O. V., Kukushkin O. V., Litvinchuk S. N., Mazepa G. A., Rozanov J. M. (2007). A case of natural triploidy in European diploid green toad (*Bufo viridis*), with some distributional records of diploid and tetraploid toads. Russ J Herpetol, 14(2), 121–132

Cavallo D., Devita R., Eleuteri P., Borkin L., Emechenko V., Odiema G., Balletto E. 2002. Karyological and flow cytometric evidence of triploid specimens in *Bufo viridis* (Amphibia Anura). Eur J Histochem, 16: 159–164

Degani G.,Goldberg T., Gasith A., Elron E., Nevo E.2013.DNA variations of the green toad *Pseudepidalea viridis* (syn. *Bufo viridis*) from various habitats. Zool Stud, 52: 1–15

Eiselt J., Schmidtler J. F. 1971. Vorlaufige Mitteilung über zwei neue Subspecies von Amphibia salientia aus dem Iran. Ann Naturhist Mus Wien, 75: 383–385

Fakharzadeh F., Darvish J., Ghassemzadeh F., Kami H. G. 2009. Anuran karyological study of Khorasan province. Asian J Bio Sci, 2(3): 66–73

Inger R. F. 1972. *Bufo* of Eurasia. In Blair W. F. (Ed.), Evolution in the genus *Bufo*. Austin: University Texas Press, 102–118

Javari M., Torki F. 2009. Notes on morphology, ecology, behavior and systematics of *Bufo luristanicus* Schmidt, 1952. Herpetozoa, 21: 171–178

Kasahara S., Siva A. P., Haddad C. F. 1996. Chromosome banding in three species of Brazilian toads (Amphibia-Bufonidae). Rev Brasil Genet, 19: 237–242

Levan A., Fredga K., Sandberg A. A. 1964. Nomenclature for centromeric position on chromosomes. Hereditas, 52: 201–220

Litvinchuk S. N., Rosanov J. M., Usmanova N. M., BorkinL. J.,Mazanaeva L. F., Kazakov V. I. 2007. Variability of microsatellites BM224 and Bcal7 in populations of green toads (*Bufo viridis* complex) differing by nuclear DNA content and ploidy. Cell Tissue Bio, 1 (1): 65–79

Masik E. J., Kadirova B. K., Toktosunov A. T. 1976. Karyotype pattern in the green toad *Bufo viridis* in Kirghizia. Zool Zh, 55(11): 1740–1743 (In Russian with English summary)

Mertens R. 1971. Zur Kennthis von *Bufo luristanicus* (Salientia Bufonidae) .Salamandra, 7: 83–84

Miura I. 1995. The late replication banding patterns of chromosome are highly conserved in genera Rana, Hyla and Bufo (Amphibia, Anura). Chromosoma, 103: 567–574

Pallas P. S., Baldinger E. G., Erxleben J. C. P. 1777. Naturgeschichte merkwürdiger Thiere, in welcher vornehmlich neue und unbekannte Thierarten durch Kupferstiche, Beschreibungen und Erklarungen erlautert werden. Berlin and Stralsund: G. A. Lange

Roth P., Ráb P. 1986. Karyotype analysis of the *Bufo viridis* group: Systematic implications. In Roèek Z. (Ed.), Studies in Herpetology. Prague: Charles University, 131–135

Schmidt K. P. 1955. Amphibians and reptiles from Iran. Vidensk Medd Dansk Naturh Foren, 117: 193–207

Schmid M. 1978. Chromosome banding in amphibia. Chromosoma, 66(4): 361–388

Schmidtler J. J., Schmidtler J. F. 1969. Über *Bufo* surdus; mit einem Schlüssel und Anmerkungen zu den übrigen Kr.ten Irans und West-Pakistans. Salamandra, 5: 113–123

Siripivasing P., Chulalaksananukul W., Pariyanonth P., Kaewsri S., Sittigul, S., Seatung N., Tanomtong A. 2008. The identification of the sex chromosome and karyotype of four toad species (genus *Bufo*) in Thailand bt T-lymphocyte cell culture. Cytologia, 73(3): 229–241

Stöck M., Günther R., B.hme W. 2001a. Progress towards a taxonomic revision of the Asian *Bufo viridis*group: Current status of nominal taxa and unsolved problems (Amphibia: Anura: Bufonidae). Zool Abh Staatl Mus Tierkunde Dresden, 51: 253–319

Stöck M., Frynta, D., Grosse, W.-R., Steinlein, C., Schmid, M., 2001b. A review of the distribution of diploid, triploid and tetraploid green toads (*Bufo viridis* complex) in Asia including new data from Iran and Pakistan. Asiatic Herp Res, 9: 77–100

Stöck M., Grosse W. R. 2003. Die *Bufo viridis*-Untergruppe in MIttel- und Zentralasien: Eine-bersichzuVerbreitung, Polyploidie, Paarungsrufen und Taxonomie. Mertensiella, 14:179–217

Stöck M., Steinlein C., Lamatsch D. K., Schartl M., Schmid M. 2005. Multiple origins of tetraploid taxa in the Eurasian *Bufo viridis* subgroup. Genetica, 124: 255–272

Stöck M., Moritz C.,Hickerson M.,Frynta D., Dujsebayeva T., Eremchenko V., Macey J.R., Papenfuss T.J., Wake D.B. 2006. Evolution of mitochondrial relationships and biogeography of Palearctic green toads (*Bufo viridis* subgroup) with insights in their genomic plasticity. Mol Phyl Evol, 41: 663–689

Zareian H., Gholamhosseini A., Esmaeili H.R., Teimori A. 2012. Vertebrates of Kuh-e-Gorm non-hunting area, Jahrom, Iran: Diversity, conservation and challenges. Iran J Anim Biosyst, 8(2): 169–182